APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1989, p. 2356–2359
0099-2240/89/092356-04$02.00/0

Vol. 55, No. 9

Detection, Growth, and Amine-Producing Capacity of Lactobacilli in Cheese

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Received 5 April 1989/Accepted 16 June 1989

A differential plating medium was developed to detect decarboxylating lactobacilli in cheese. With this medium, 15 cheeses made from raw milk were investigated for the presence of these bacteria. Five histidine-decarboxylating strains and one tyrosine-decarboxylating strain were isolated. The isolates were identified with the API 50L system. Accordingly, each of the five histidine-decarboxylating strains was identified as *Lactobacillus buchneri*, whereas the tyrosine-decarboxylating strain is a representative of *Lactobacillus brevis*. Cheesemaking experiments using a low inoculum concentration of the histidine-decarboxylating *L. buchneri* strain St2A (0.2 CFU/ml of milk) showed that, under conditions of accelerated proteolysis, histamine may accumulate rapidly; after 3 months of ripening, 410 mg/kg was found. An inoculum concentration of 5 CFU/ml gave rise to the formation of 1,060 mg/kg.

For many different foods, the formation of biogenic amines may be a problem, since these compounds may cause food poisoning (2, 18, 23). In general, the illness is a mild one, and the symptoms usually disappear within a few hours, but serious cases have also been reported (20, 25). Usually, amine production results from the presence of bacteria that are capable of decarboxylating amino acids (9). In particular, histamine formation in fish has received much attention (1, 8), but fermented foods such as cheese also sometimes contain high concentrations of biogenic amines (3, 4, 12, 16, 27).

In fish, amines are predominantly formed by the gram-negative bacteria, particularly members of the family *Enterobacteriaceae*. Histidine-decarboxylating strains were found among the genera *Proteus*, *Klebsiella*, *Hafnia*, and *Enterobacter* (26). In cheese, amines can also be produced by members of the family *Enterobacteriaceae* (14), but investigations with Dutch types of cheese revealed that, under normal conditions, these bacteria do not grow to high densities and also die off quickly. In most of the cases in which large amounts of amines have been formed in cheese, decarboxylating lactobacilli are probably responsible (11). Previous studies revealed that such strains are particularly found among mesophilic heterofermentative species (5, 14, 24). However, in addition to decarboxylase activity the precursor concentration (viz., the free amino acids) is important (13), and toxic amounts of amines can be found only in cheese that has undergone excessive proteolysis (11).

In recent years, considerable research has been undertaken to accelerate the ripening of cheese (6, 7, 17). Generally, accelerated ripening is accompanied by an intensified proteolysis, thus rendering the cheese more susceptible to amine formation. Moreover, there is a growing interest in the possible toxicity of low doses of biogenic amines (22). Therefore, there is a greater need for a simple method to detect amine-producing lactobacilli in cheese. Niven et al. (21) described a plate method which is suitable for the detection of histidine-decarboxylating bacteria in fish and fish products, but it proved to be of limited value for cheese (14). We modified this method to detect histamine-producing lactobacilli in cheese. Furthermore, we investigated whether this method could also be used to detect lactobacilli that produce other biogenic amines.

In previous studies, histamine formation was studied only in cheese containing 10^8 CFU of the decarboxylating *L. buchneri* strain St2A per g (11, 13, 14). For this strain, 10^8 CFU/g is probably the maximum attainable density in cheese, resulting from the presence of more than five lactobacilli per milliliter of milk. In common practice, this density is rarely observed. However, since densities on the order of 10^6 to 10^7 CFU/g occur more often, it is important to know the potential for histamine formation in these cases, especially if proteolysis is accelerated. Another set of cheesemaking experiments was therefore set up, in which the milk used contained only 0.01, 0.2, or 5.0 CFU of strain St2A per ml.

MATERIALS AND METHODS

Cheesemaking. The cheese was made from pasteurized milk by using the standard procedure for Gouda cheese production in 200-liter batches. To accelerate proteolysis, a concentrated suspension of heat-treated starter cells with a low souring activity was added to the milk together with the regular starter, which consisted of *Lactococcus* and *Leuconostoc* species (6). Decarboxylating bacteria (*L. buchneri* St2A) were added after pasteurization of the cheese milk.

Cultures. *L. buchneri* St2A was kindly provided by S. L. Taylor, University of Nebraska, Lincoln. The homofermentative *Lactobacillus* strain NZK1 was isolated from a fermented sausage and was capable of decarboxylating both tyrosine and ornithine. The lactobacilli were propagated in sterilized litmus milk supplemented with 1% yeast extract and 1% glucose.

Samples. The applicability of the newly developed media was investigated with 15 samples of Gouda-type cheese. These cheeses were all made from raw milk and were obtained from various manufacturers.

Chemical analysis. Analysis for biogenic amines in cheese was performed by high-performance liquid chromatography (HPLC) as previously described (15). The concentration of individual free amino acids was determined with an LKB model 4151 Alpha-plus amino acid analyzer as previously described (11).

Bacteriological analyses. The number of mesophilic lacto-

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TABLE 1. Biochemical characterization of decarboxylating lactobacilli isolated from cheese by using the API 50L system[a]

Strain	API 50L system test result[b]																
	L-Arabinose	Ribose	D-Xylose	Galactose	Glucose	Fructose	Esculin-iron-citrate	*N*-Acetyl-glucosamine	Maltose	Lactose	Melibiose	Sucrose	Melezitose	D-Raffinose	Gluconate	5-Ketogluconate	α-Methylglucoside
NZHD1	+	+	−	±	+	±	+	−	+	−	±	+	+	−	±	±	−
NZHD2	+	+	−	±	+	±	+	−	+	−	+	+	+	±	±	±	−
NZHD3	+	+	−	±	+	±	+	−	+	−	±	+	+	+	±	±	−
NZHD4	+	+	±	±	+	±	+	−	+	+	+	+	+	±	±	±	−
NZHD5	+	+	±	±	+	±	+	−	+	+	+	+	+	±	±	±	±
NZTD1	+	+	+	±	±	±	+	±	±	+	±	−	−	−	±	±	−
St2A	+	+	±	+	+	+	+	−	+	±	+	+	+	+	±	±	−

[a] The following reactions were negative for all the strains: glycerol, erythritol, D-arabinose, L-xylose, adonitol, β-methylxyloside, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methylmannoside, amygdalin, arbutin, salicin, cellobiose, trehalose, inulin, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, and 2-ketogluconate.

[b] ±, Weakly positive reaction.

bacilli present in cheese was estimated by using tryptone-yeast extract-beef extract agar (TGV 5.4) (10) after 5 days of incubation at 30°C.

The medium used to detect decarboxylating bacteria contained 0.5% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), 0.5% NaCl, 0.1% glucose, 0.05% Tween 80, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCO_3$, 0.006% bromocresol purple, 0.005% $MnSO_4 \cdot 4H_2O$, 0.004% $FeSO_4 \cdot 7H_2O$, 2% agar, and a 2% concentration of a particular precursor amino acid (histidine, tyrosine, lysine, or ornithine). After sterilization (at 121°C for 10 min), the pH was 5.0 ± 0.1. Decimal dilutions of the cheese samples were incubated anaerobically at 30°C for 7 days.

Decarboxylating isolates were identified on the basis of the catalase test, microscopy (morphology), the Gram stain reaction, and carbohydrate fermentations. The latter tests were done with the API 50L *Lactobacillus* identification system (API System S.A., La Balme les Grottes, Montalieu Vercieu, France) (see Table 1). To distinguish between homo- and heterofermentative organisms, HHD medium was used (19).

Confirmation of the amine-forming capacity. The decarboxylating strains were inoculated into MRS medium (5a) supplemented with 0.25% each of the amino acids histidine, tyrosine, lysine, and ornithine. After 16 and 28 h of incubation at 30°C, 3 ml of culture fluid was mixed with 3 ml of 10% trichloroacetic acid and centrifuged for 10 min at 10,000 × *g* and the supernatant was analyzed for the presence of biogenic amines by HPLC (15).

RESULTS AND DISCUSSION

Optimization of the composition of the differential plating medium. The basal composition of the differential plating medium was given by Niven et al. (21). To enhance the growth of lactobacilli, some metal sulfates (Mg, Mn, and Fe) and Tween 80 were added. Colonies of decarboxylating bacteria should have been detectable by the presence of a purple halo. However, by using this composition only a weak color change in the medium surrounding the colonies was observed. Contrast was improved by lowering the initial pH of the medium to 5.0 and by adding glucose to the medium. An optimal glucose concentration of 0.1% was established. When higher concentrations were used, homofermentative strains such as *Lactobacillus* strain NZK1 produced too much acid, thereby counteracting the purple discoloration. With these adaptations, agar media with one of the three amino acids, i.e., histidine, lysine, and ornithine, were prepared. Growth of decarboxylating bacteria was easily recognizable because of their purple halo; nondecarboxylating strains produced a yellow halo.

Tyrosine has a very low solubility, and at the 2% tyrosine concentration plates were translucent and the characteristic color change could not be used to detect tyrosine-decarboxylating lactobacilli. However, tyrosine-decarboxylating bacteria were surrounded by a clear halo, resulting from the disappearance of the tyrosine sediment.

Identification of some decarboxylating bacteria from cheese. Fifteen samples of cheese were investigated for the presence of decarboxylating bacteria with the above-described media. As could be expected with cheese made from raw milk, various samples contained amine-producing bacteria. Five histidine-decarboxylating strains and one tyrosine-decarboxylating strain were isolated. The amine-forming capacity was confirmed by HPLC analysis. The rate of production in the supplemented MRS medium was of the same order of magnitude as that by *L. buchneri* St2A (data not shown).

All strains consisted of gram-positive, catalase-negative, and facultatively anaerobic rods with a heterofermentative metabolism. The histidine-decarboxylating strains NZHD1, NZHD2, NZHD3, NZHD4, and NZHD5 showed great similarity in their sugar fermentation patterns (Table 1), although they were all isolated from different cheeses. Moreover, they were also very similar to the well-known histamine-producing *L. buchneri* strain St2A, which was isolated in the United States from a histamine-rich Swiss-type cheese (24). These results strongly suggest that these newly isolated strains also belong to the species *L. buchneri*. Therefore, it is concluded that notorious histamine producers are usually found among this species.

The sugar fermentation pattern of NZTD1 (Table 1), a tyrosine-decarboxylating strain, was quite similar to that of the histidine-decarboxylating isolates, but because melezitose was not fermented NZTD1 most likely is a representative of *L. brevis*. It was shown earlier that within this

FIG. 1. Growth of *L. buchneri* St2A in cheese. Three lots of milk were pasteurized, and after addition of the regular starter, the histidine-decarboxylating strain St2A was inoculated at 0.01 (a), 0.2 (b), or 5 CFU/ml (c). Outgrowth of these lactobacilli in the cheese was monitored during 26 weeks of ripening.

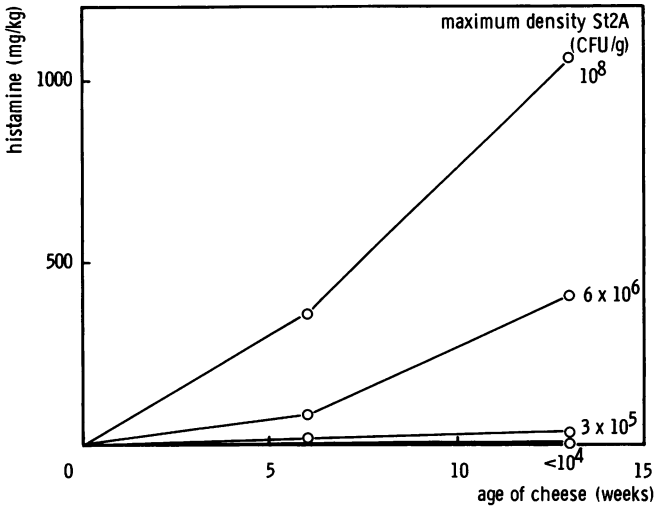

FIG. 2. Formation of histamine in cheese by *L. buchneri* St2A. Cheese, in which the histidine-decarboxylating strain St2A reached maximum densities of 3×10^5, 6×10^6, and 10^8 CFU/g, was examined for its histamine content during 13 weeks of ripening. A control cheese without St2A ($<10^4$ CFU/g) was also investigated.

species more tyramine producers can be found (5, 14). It would be interesting to investigate whether the capacity to produce histamine or tyramine is species dependent.

Growth and amine-forming capacity of *L. buchneri* St2A in cheese with accelerated ripening. A cheesemaking experiment was set up in which pasteurized milk was contaminated with the histidine-decarboxylating strain St2A. In a previous study (14), the presence initially of only a few lactobacilli per milliliter resulted in 10^8 CFU/g in the cheese after 4 to 6 weeks of ripening. Adding more lactobacilli to the milk yielded about the same maximum density. To obtain lower maximum densities in this experiment, 0, 0.01, 0.2, or 5 CFU/ml was added to the milk in four different cheese vats. Figure 1 shows the growth of these bacteria during cheese ripening. Mesophilic lactobacilli other than strain St2A were not found in the cheeses, and in the control cheese no lactobacilli were detected at all (detection limit, 10^4 CFU/g). The results demonstrate clearly that this strain can grow very well in cheese. Assuming that all the added bacteria are trapped in the curd, it follows that contamination with only one microorganism eventually leads to more than 10^6 CFU in the cheese after several weeks of ripening. After reaching the maximum count, the density remained fairly stable until at least 26 weeks of ripening.

In Fig. 2, the histamine formation in these cheeses is illustrated. The control cheese without St2A did not contain histamine (<10 mg/kg). After 3 months of ripening, the cheese with about 3×10^5 CFU/g contained 35 mg/kg, the cheese with 6×10^6 CFU/g contained 410 mg/kg, and the cheese with 10^8 CFU/g contained 1,060 mg/kg. In a previous study, a kinetic model for histamine formation in Gouda cheese was proposed (13). This model was based on the histidine decarboxylase activity, which was determined in a heavily contaminated Gouda cheese, and on the rate of histidine liberation, which was measured in an uninfected control cheese. If the model is used to predict amine production in the experimental cheese described above, the accelerated precursor formation rate has to be taken into account. Amino acid analysis of the control cheese revealed that after 3 months of ripening the free histidine content was 1,400 mg/kg, whereas normal Gouda cheese after the same ripening period contains less than 200 mg/kg (11). Assuming that 1,400 mg/kg was also released in the cheese contaminated with St2A, it follows that the predicted amine concentrations agree well with those actually found.

From the results, a more general conclusion can be drawn; whereas for normal Gouda cheese 10^7 CFU of decarboxylating lactobacilli per gram do not really present a health hazard, in cheese containing high levels of free amino acids such a density is certainly too high. Since a density of 10^7 CFU/g may result from the presence of only three lactobacilli per 10 ml of milk, much care should be given to the maintenance of good manufacturing practice during the production of such cheese. In this respect, the use of the selective medium to detect decarboxylating bacteria may prove to be a valuable tool.

ACKNOWLEDGMENT

We are grateful to M. Twigt for technical assistance.

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